EFFECTS OF AQUATIC CONDITIONING ON CARTILAGE AND BONE METABOLISM IN YOUNG HORSES

A Thesis

by

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ABSTRACT

Potential for aquatic exercise to affect cartilage and bone metabolism in young horses was investigated. Thirty Quarter Horse yearlings were stratified by age, body weight (BW), and sex and randomly assigned to one of three treatments for 140-d to compare effects of aquatic vs. dry exercise on bone and cartilage metabolism in young horses transitioning to an advanced workload. Treatments included non-exercise control (CON; n=10), dry treadmill (DRY; n=10), or aquatic treadmill exercise (H2O; n=10; water at 60% of wither height, WH). Horses were housed individually (3.6×3.6m) from 0600 to 1800, allowed turnout (74×70m) from 1800 to 0600. During Phase I (d 0-112), DRY and H2O walked on treadmills 30 min/d, 5 d/wk. In Phase II (d 113-140) all exercise horses transitioned to an advanced workload 5 d/wk. Every 14-d, WH, hip height (HH), and BW were recorded. Left third metacarpal radiographs on d 0, 112, and 140 were analyzed for radiographic bone aluminum equivalence (RBAE). At 28-d intervals, serum samples were collected to quantify concentrations of osteocalcin and C-telopeptide crosslaps of type I collagen (CTX-1) and synovial fluid samples were collected to quantify concentrations of prostaglandin E_2 (PGE_2), collagenase cleavage neopeptide (C2C), collagenase of type I and type II collagen (C1,2C), and carboxypeptide of type II collagen (CPII) using ELISAs. Data were analyzed using PROC MIXED of SAS. There were treatment × day interactions ($P<0.01$) where OC and CTX-1 remained consistent in both exercise groups while inconsistently increasing in CON. There were no treatment differences ($P>0.30$) in RBAE, BW, or HH, but all increased over time ($P<0.01$). There were no treatment × day

interactions of synovial inflammation or markers of cartilage metabolism, however there was an effect of day for each marker ($P<0.03$). Changes in biomarkers of cartilage turnover in horses exercised at the walk, whether dry or aquatic, could not be distinguished from horses with turnout alone. This study indicates that early forced exercise supports consistent bone metabolism necessary for uniform growth and bone development, and that there are no negative effects of buoyancy on cartilage metabolism in yearlings transitioned from aquatic exercise to a 28-d advanced workload.

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NOMENCLATURE

BW – Body Weight

C1,2C – Collagenase of Type I and Type II Collagen

C2C – Collagenase Cleavage Neopeptide

CON – Control Group

CPII – Carboxypeptide of Type II Collagen

CTX-1 – C-Telopeptide Crosslaps of Type I Collagen

DRY – Dry Treadmill Exercise Group

H2O – Aquatic Treadmill Exercise Group

HH – Hip Height

PGE_2 – Prostaglandin E_2

RBAE – Radiographic Bone Aluminum Equivalence

WH – Wither Height

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CHAPTER I

INTRODUCTION

To achieve a level of competitiveness in a young animal's performance career, it is a common practice in the equine industry to enroll horses in an exercise-training program at a young age (12-18 mo). This time frame coincides with a period of growth that is characterized by adaptation of both bone and soft tissue structures. Early career starts have been associated with career longevity, which is likely due to strengthening by early stimulation of the musculoskeletal system (Rogers et al., 2012). Cartilage adaptation early in life is essential for a well-structured articular cartilage collagen network, as cartilage remains fairly static once mature (Brama et al., 2000). In contrast, bone undergoes remodeling throughout the life of the animal but is only capable of modeling during early stages of life. During modeling, osteoclasts resorb bone from areas under minimal stress while osteoblasts regulate bone deposition in areas subjected to greater force, allowing for net bone formation (Langdahl et al., 2016). This period of growth may be used to maximize musculoskeletal adaptation and development, which may improve athletic career and decrease the incidence of musculoskeletal-related injury (Barneveld and van Weeren, 1999).

Recently, the equine industry has seen a rise in popularity of aquatic treading. Buoyancy in aquatic therapy lifts and reduces axial loading on articular joints by minimizing vertical ground forces (King et al., 2013). While decreased load on bones may be beneficial in rehabilitation cases to reduce stress on damaged bone, this

technology is also being used to prepare young horses for sales and future performance. When transitioned into a high intensity exercise program on dry land, lack of skeletal adaptation for increased mechanical loading may lead to a higher degree of breakdown of bone and cartilage.

Conditioning and training of performance horses involves many factors such as behavior modification, cardiovascular fitness and skeletal strength. Of these factors it is most difficult to assess strength of the skeletal framework until a physical symptom is observed when an injury arises (Nielsen et al., 1997). Biomarkers in serum and synovial fluid have potential to detect subtle or early changes to tissues before clinical signs are evident (McIlwraith, 2005). Biomarkers relative to bone and cartilage turnover may provide valuable insight to the effect of exercise in the juvenile horse. Such metabolic biomarkers (Table 3) include: osteocalcin, a serum biochemical marker of bone formation; C-telopeptide crosslaps of type I collagen (CTX-1), a serum biochemical marker of bone degradation; synovial prostaglandin E_2 (PGE_2), a marker of joint inflammation; catabolic collagenase cleavage neopeptide (C2C) and collagenase of type I and type II collagen (C1,2C) indicative of cartilage degradation; and anabolic carboxypeptide of type II collagen (CPII) which increases in an effort to repair articular cartilage (LePage et al., 2001). Therefore, the objectives of this study were to determine the influence of early forced exercise and type of exercise on serum and synovial fluid biomarkers of young horses, and to provide insight into the effects of conditioning on joint inflammation and cartilage/bone turnover. Furthermore, to investigate the effects of differing conditioning programs on joint inflammation, cartilage metabolism, and bone

mineralization when transitioned from a moderate to an advanced workload on a dry surface.

CHAPTER II

REVIEW OF THE LITERATURE

Bone and Articular Cartilage Structure

The equine musculoskeletal system functions to provide support, protect internal organs, and allow for locomotion. Because horses are athletic animals, locomotion is a function of particular importance. Bones provide rigid structure and protection, while joints allow for movement of these bones against one another and provide lubrication to minimize friction. Synovial fluid is the primary lubricant in the synovial cavity of the joint, where bones meet, and is considered to be an ultrafiltrate of plasma (Steel, 2008). Synovial fluid is filtered through the synovial membrane, allowing it to contain lubricant molecules secreted by synoviocytes and chondrocytes as well as some nutrients. During a state of disease, biomarkers released from the articular cartilage may be present in synovial fluid.

Articular cartilage lies between the synovial cavity and the subchondral bone. Articular cartilage is unique compared to many tissues due to its lack of vasculature, nerves, or lymphatics and therefore must receive nutrients provided through the synovial fluid. The extracellular matrix is composed of water, collagen, proteoglycans, and lesser amounts of noncollagenous proteins and glycoproteins (Fox et al., 2009). Additionally, chondrocytes are a specialized cell found dispersed throughout the articular cartilage. Chondrocytes are responsible for producing the components of the extracellular matrix, and hypertrophy of these cells can lead to cartilage degradation (Akkiraju and Nohe,

2015). Articular cartilage contains both non-calcified and calcified regions; non-calcified cartilage can be further divided into three zones, all of which have different composition dependent upon depth from the synovial cavity. As cartilage gets deeper, chondrocyte structure and collagen orientation differ. Furthermore, articular cartilage varies by site within the joint which is influenced by the load-bearing distribution throughout different joints (Xia et al., 2018).

The calcified zone of cartilage is adjacent to the thin layer of subchondral bone, which is responsible for providing structural support for the articular cartilage. Subchondral bone consists of both trabecular (or cancellous) bone and osteonic (or cortical) bone. Cancellous bone comprises the epiphysis, metaphysis, and bone marrow compartment of bones and has a honeycomb like structure, while cortical bone is dense and solid and makes up the diaphysis (Clark, 2008). Aside from providing support and locomotion, bone also produces many growth factors and cytokines which regulate growth and remodeling of both bone and cartilage.

Bone and Joint Health

To achieve a level of competitiveness in futurities or to prepare young horses for sales, it is common practice in the equine industry to enroll horses in an exercise-training program at a young age (around 12 to 18 mo of age). Common early training practices generally include three to four months of slow to moderate training that is followed by one to two months of skill training specific to discipline. During this time period, horses are still growing, and their musculoskeletal system is still being developed. Due to the

dynamic nature of bone and cartilage during growth, the goal of this time period should be to maximize musculoskeletal strength, and thereby minimize wastage.

Growth is characterized by bone modeling, a process by which bone formation and resorption occur in an uncoupled manner (Langdahl et al., 2016). This time period is of particular interest because bone is most capable of reacting to external forces during modeling (Bartl and Bartl, 2017). Exercise places forces on bones and induces modeling in young horses. During this process osteoblasts lay down bone in areas that are receiving higher forces, while osteoclasts resorb bone from areas that are not under as much stress. Contrastingly, remodeling occurs in bone throughout the life of the animal. Remodeling occurs when osteoclasts and osteoblasts act in a coupled manner, and serves mainly to replace older, microdamaged bone with new, healthier bone (Clark, 2008). In young horses, it is difficult to distinguish whether changes in bone are specifically due to modeling or remodeling, but this is a time of great importance for bone development. Loading during growth allows the bone to adapt to a specific type of exercise to become stronger. Exercise, specifically galloping (≥14 m/s) has been shown to increase bone density and bone size in two and three-year-old Thoroughbreds, leading to an increased bone strength index (Firth et al., 2012).

Bone mineral density has been shown to increase in horses in the first year of life when allowed free exercise in the pasture and even more so in horses who underwent a sprinting exercise (Brama et al., 2009). Furthermore, bone was shown to adapt more at a location central to loading compared to a more peripheral, marginally loaded site. These studies indicate that introduction into early training programs may increase bone density

and minimize risk for bone damage, as more dense bone has demonstrated the ability to resist fatigue that may cause injury (Martig et al., 2020).

In addition to bone formation and health, regular joint loading is essential for a strong and well-organized articular cartilage collagen network. Foals are born with uniform cartilage which then begins to differentiate based on weight bearing within the joint. Adaptation early in life is beneficial for the final quality of the cartilage, which remains fairly static once maturity is reached (Brama et al., 2000). Collagen components have been observed to have reached mature biochemical composition by six months of age, as compared to the approximately four years it takes bone (van der Harst et al., 2005). Furthermore, cartilage appears to adapt differently within the joint depending on variations in load within the joint, by increasing chondrocytes or chondrocyte activity and proteoglycan synthesis in high activity areas, allowing for increased ability to resist mechanical stress (Little and Ghosh, 1997). High intensity exercise in 3-5-year-old Standardbreds has been shown to alter cartilage metabolism in a manner consistent with collagen network loosening, potentially predisposing the joint to damage (Little et al., 1997). However, non-strenuous exercise in young horses appears to only temporarily increase glycosaminoglycan content, and not collagen (Brama et al., 1999). Caution should be exercised, as repeated stress and trauma may lead to damage to the articular surface and contribute to an increased risk for degenerative joint disease development later in life (Billinghurst et al., 2003).

Aquatic treading is becoming increasingly popular as a method of rehabilitative therapy as well as conditioning for young horses. Aquatic conditioning is known to

increase cardiovascular and muscular endurance in humans (Chu et al., 2004; Lee and Oh, 2014) Although changes due to conditioning over time has not been well investigated in the horse, addition of water at stifle level has been shown to increase heart rate in horses (59 bpm to 69 bpm) as compared to control when exercising at the same speed (Greco-Otto et al., 2017). However, the effects of aquatic treadmill exercise on human and equine bone and joint are not well defined. By placing the horse in water, buoyancy is increased. Buoyancy in aquatic therapy is a force that lifts and reduces axial loading on the joints by minimizing vertical ground reaction forces (King et al., 2013). Mice exposed to partial weight bearing have shown a decrease in bone mineral density with a 20% reduction in weight bearing (Ellman et al., 2013). While this decreased load on bones may be beneficial in rehabilitation cases to reduce stress on damaged bone, it may not be positive when fitting young horses for entering training programs. By decreasing loading on the bones, a decreased degree of turnover may be observed. When transitioned into a high intensity exercise program on dry land, lack of metabolism to prepare for an increased workload may lead to increased breakdown of bone and cartilage.

Serum and Synovial Fluid Markers

Conditioning and training of performance horses involves many factors such as behavior modification, cardiovascular fitness and skeletal strength. Of these factors it is most difficult to assess strength of the skeletal framework until a physical symptom is observed when an injury arises (Nielsen et al., 1997). Biomarkers in serum and synovial fluid have potential to detect subtle or early damage to tissues before clinical signs are evident. Biomarkers relative to cartilage turnover may provide valuable insight to the

effect of exercise in the juvenile horse. These synovial metabolic biomarkers include prostaglandin E_2 (PGE_2), catabolic collagenase cleavage neopeptide (C2C) and collagenase of type I and type II collagen (C1,2C), and carboxypeptide of type II collage (CPII). As a marker of joint inflammation, elevated PGE_2 can be indicative of joint diseases, and can cause articular cartilage damage and reduce repair (Gibson et al., 1996). When damage to the cartilage occurs, catabolic C2C and C1,2C are released into the synovial fluid. Biomarkers of type II collagen and overall collagen degradation, respectively, an associated increase in these markers has been observed following an insult to the joint (Wang et al., 2015). A marker of collagen formation, CPII is released in an effort to repair articular cartilage (LePage et al., 2001). When synovial CPII levels are higher than those of C2C, it is indicative of net formation of cartilage. Through the use of synovial fluid biomarkers, it is possible to detect early changes in articular cartilage damage.

Bone degradation and reformation due to modeling and remodeling in response to exercise is a point of interest, but generally cannot be measured directly as euthanization of horses is not practical in most cases. Therefore, serum biomarkers and interpreting radiographic scans can be used to assess changes in bone. Osteocalcin, a small non-collagenous protein, is produced by osteoblasts (Lepage et al., 2001; Zoch et al., 2016). Osteocalcin is a serum biochemical marker that is well accepted as a marker of bone formation (LePage et al., 2001). CTX-1, a serum marker of bone resorption, has been utilized in horses (Delguste et al., 2007). In combination osteocalcin and CTX-1 can be utilized to give an overall picture of bone turnover. Methods of assessing bone mineral

density such as quantitative computed tomography and bone breaking strength have been utilized in horses (Yamada et al., 2015; Tóth et al., 2013), however these methods typically require euthanasia in order to be practical. One method of non-invasively assessing bone mineral density is through radiographic bone aluminum equivalence (RBAE). In this method, a radiographic scan of the bone is taken and through a computer analysis is compared with a standardized step wedge (LePage et al., 2001). Osteocalcin has been shown to have a correlation with bone scans (Sharif et al., 1995), which indicates that osteocalcin in conjunction with bone scans can help track changes in bone development.

Currently, there are critical gaps in knowledge in relation to the influence of early forced exercise and type of exercise on serum and synovial fluid biomarkers of young horses, and the effects of conditioning on joint inflammation and cartilage/bone turnover. Furthermore, the effects of differing conditioning programs on joint inflammation, cartilage metabolism, and bone mineralization when transitioned from a moderate to an advanced workload on a dry surface have not been well investigated.

CHAPTER III

MATERIALS AND METHODS

All procedures and handling of horses were approved by the Texas A&M University Institutional Animal Care and Use Committee (2017-0376).

Horses and Treatments

Thirty yearling Quarter horses (343 ± 28 kg; 496 ± 12 d of age; 15 geldings and 15 fillies) of similar breeding were stratified by body weight (BW), age, and sex and randomly assigned to one of three treatment groups during the 140-d trial (Table 1). Treatments consisted of non-exercised controls (CON; n=10), treadmill-exercised horses (DRY; n=10), and aquatic treadmill-exercised horses (H2O; n=10). Prior to the start of the trial, radiographs of both radial carpal joints were performed by Texas A&M University Equine Field Services (College Station, TX). All horses determined to be radiologically normal were included in the study and fed 1.25% BW (as-fed) commercial concentrate feed (SafeChoice Mare and Foal, Nutrena, Minneapolis, MN). Concentrate was offered in two equal feedings, individually at 0600 and 1800, and all horses were allowed *ad libitum* access to coastal bermudagrass hay (*Cynodon dactylon*). Diets were formulated to meet or slightly exceed nutrient requirements for young, exercising horses undergoing rapid growth (NRC, 2007). Throughout the trial, horses were housed in individual stalls (3.6 m x 3.6 m) and allowed turnout (74 m x 70 m) for approximately 10 h/d to mimic housing conditions for young horses entering into training programs.

Exercise Protocol

Non-exercised CON received no forced exercise for the duration of the study. Prior to d 0, exercise groups were familiarized (5 d) with each treadmill apparatus (HorseGym USA, Wellington, FL) until they were accustomed to loading onto and walking on the treadmill. The acclimation period included each H2O horse walking in and out of the treadmill, as well as walking into the unit, and adding and draining water in order to acclimate each horse to the sound of the unit (Adair, 2011). Exercise protocol for horses assigned to DRY and H2O was divided into two phases. Phase I (d 0-112) represented a long-term submaximal exercise program, mimicking industry standards for sales fitting programs, where DRY and H2O groups were exercised 5 d/wk for 21 min at the walk, increasing 5 min/wk until a total time of 30 min was reached and maintained throughout the remainder of the study. Phase I began with horses walking 1.2 m/s and 0.2 m/s was added every 28 d until the end of Phase I to reach a maximum speed of 1.8 m/s. The water treadmill took 8 min to fill and 8 min to drain, and water level was maintained at 60% of wither height (WH) throughout treadmill exercise. Water temperature averaged 27 °C throughout the project to maximize comfort (Adair, 2011). The transition to phase II began on d 113 in which both DRY and H2O groups exercised in a circular, free stall exerciser (Table 2; Priefert Manufacturing, Mt. Pleasant, TX) to evaluate bone and cartilage responses to patterns of strain following their respective conditioning programs to completion of the study on d 140.

Sample Collection

Growth measurements were recorded every 14 d, and included BW, WH, and hip height (HH). Measures including, HH and WH, were obtained by the same individual at every timepoint using an altitude stick (Sullivan Supply, Inc., Hillsboro, TX). Synovial fluid and serum samples were obtained every 28 d for the entire 140-d trial. Sampling occurred Fridays following exercise, which allowed 2 d to recover before exercise began again the following Monday. Samples were collected at the same time every sample day to account for potential diurnal variation. Blood was collected via jugular venipuncture into 10-ml additive-free sterile blood collection tubes (BD Vacutainer, Franklin Lakes, NJ). Blood samples remained at room temperature for approximately 1 h prior to centrifugation at 2000 × *g* at 10 °C for 20 minutes (ALC, PM140R, Thermo Fisher Sci., Waltham, MA) to harvest serum. Synovial fluid was collected via sterile arthrocentesis of the right radial carpal joint by a board-certified veterinary surgeon from the Texas A&M University Large Animal Clinic (College Station, TX).

Horses were sedated using xylazine HCl, which was administered intravenously at recommended dosages. The carpal joint was aseptically aspirated from a location medial to the extensor carpi radialis tendon in the palpable depression between the radial carpal bone and the third carpal bone, to a depth of approximately 12.7 mm to avoid unnecessary contact with articular cartilage (McIlwraith and Trotter, 1996). Synovial fluid was transferred to 10-ml additive-free tubes, and immediately placed on ice. All samples were stored at -80 °C for later analysis. On d 0, 112, and 140, dorsal-palmar radiographs of the third metacarpal bone were performed by Texas A&M Equine Field

Services for later analysis. Digital radiographs were obtained using a Minxray T90 generator and a VetRocket DR Processor. Images were taken at a focal distance of 71 cm and exposure of 70 kVp and 0.08 ms. Radiographs were standardized by using a cassette holder and taking each radiograph equidistant from the bone. An aluminum (Al) stepwedge penetrometer of 11 steps ranging from 5 mm to 35 mm in 3 mm increments was placed in the same plane as the bone.

Sample Analysis

Synovial fluid samples were used to evaluate markers of joint inflammation, cartilage metabolism, and bone turnover. Synovial fluid collected throughout the trial was analyzed for PGE_2, C1,2C, C2C, and CPII using commercial ELISA kits previously validated for use in the horse (Bertone et al., 2001; de Grauw et al., 2006). Synovial fluid was plated directly for analysis of PGE_2 and C1,2C, and diluted 1:2 and 1:4, respectively for C2C and CPII. Final concentrations of these markers were read using a microplate reader with optical density set at 450 nm (BioRad 680 Microplate Reader, BioRad Laboratories, Hercules, CA). Mean minimum detectable levels of PGE_2, C2C, C1,2C, and CPII were 30.9 pg/mL, 10 ng/mL, 50 ng/mL, and 0.03 μg/mL, respectively.

Serum concentrations of osteocalcin were determined using a commercial competitive immunoassay (MicroVue Osteocalcin; Quidel Corporation, San Diego, CA). Serum samples were diluted 1:5 and final concentrations were read using a microplate reader with optical density set at 405 nm. Mean minimum detectable level of osteocalcin was 0.45 ng/mL. Serum concentrations of CTX-1 were determined using an enzyme immunological test (Serum CrossLaps (CTX-1) ELISA; Immunodiagnostic Systems,

Ltd., Gaithersburg, MD). Serum samples were plated directly, and final concentrations were read using a microplate reader with optical density set at 450 nm. Mean minimum detectable level of CTX-1 was 0.020 ng/mL. Intra-assay coefficients of variability (CVs) were established as acceptable at ≤ 15% for PGE_2, ≤ 12% for CTX-1, ≤ 10% for cartilage markers, and ≤ 7% for osteocalcin. Dorsal-palmar radiographs of the left third metacarpal were analyzed for radiographic bone aluminum equivalence (RBAE) using Quantity One (Bio-Rad Laboratories, Inc., Hercules, CA) as described by O'Connor-Robison and Nielsen (2013).

Statistical Analysis

Data were analyzed using the PROC UNIVARIATE method of SAS (SAS Inst. Inc., Cary, NC) to determine normality. All non-normal data (PGE_2) were log transformed to establish normality. Subsequently, data were analyzed using the PROC MIXED method of SAS. The model contained effects for treatment, day, and treatment × day interactions to determine the effects of various exercise treatments on cartilage and bone metabolism markers. The model contained a random effect of horse within treatment and a repeated effect of day. All biomarkers were run with d 0 as a covariate to account for baseline treatment differences. Significance was defined for all variables when $P \leq 0.05$, and a trend toward significance was established at $P \leq 0.10$.

CHAPTER IV

RESULTS

Growth Parameters

There was no influence of exercise treatment on BW ($P > 0.67$; Fig. 1). However, there was an influence of time ($P < 0.01$) as all horses, regardless of treatment, gained BW over the 140-d trial. Wither height tended to have a treatment × day interaction ($P < 0.08$; Fig. 2) characterized by a difference in response in the first 28 d of the study and HH was not affected by treatment ($P > 0.12$; Fig. 3), but all measurements increased over time ($P < 0.01$).

Markers of Inflammation and Cartilage Metabolism in Synovial Fluid

There were no treatment × day interactions on synovial inflammation and markers of cartilage metabolism ($P > 0.21$)*;* however, there was an effect of day for each of the selected biomarkers ($P < 0.03$). Synovial PGE_2 concentrations were not influenced by treatment; nevertheless, concentrations in all treatment groups tended to be lower on d 56 ($P < 0.08$; Fig. 4) when compared to other days throughout the project. Catabolic C2C decreased from d 28 to d 56 ($P < 0.01$; Fig. 5), then increased to completion of the study at d 140 ($P < 0.01$). Synovial fluid concentrations of CPII decreased from d 28 to d 56 ($P < 0.01$; Fig. 6) and increased at each time point from d 56 to d 140 ($P < 0.05$). Synovial fluid C1,2C concentrations decreased from d 28 to d 56 ($P = 0.01$; Fig. 7) and plateaued throughout the remainder of Phase I exercise, before increasing in response to Phase II ($P < 0.01$).

Serum Biomarkers of Bone Metabolism

A treatment × day interaction was observed for osteocalcin and CTX-1 where concentrations remained consistent in both DRY and H2O treatment groups throughout the 140-d project compared to CON which increased in an incongruent fashion ($P < 0.01$; Fig. 8 and 9). While a molecular response was observed through osteocalcin and CTX-1, RBAE analysis of radiographic images were not influenced by treatment ($P > 0.50$; Fig. 10 and 11); although, there was an effect of day in which both medial and lateral aspects increased in bone optical density throughout the trial ($P < 0.01$).

CHAPTER V

DISCUSSION

This study assessed the effects of aquatic and dry treadmill exercise on bone and joint metabolism in young horses. Growth measurements including WH, HH, and BW of the yearling horses increased throughout the 140-d study. This was expected as these horses were fed to meet or exceed requirements of growing horses on a rapid plane of growth, in light exercise (NRC 2007) and they have not yet attained their mature size at one year of age.

Serum osteocalcin is a previously established marker of equine bone formation produced by osteoblasts, which are responsible for deposition of bone (Nielsen et al., 1998: Billinghurst et al., 2003). In the current study, osteocalcin concentrations remained consistent in both exercise groups throughout the study. However, the CON group had an incongruent increase in osteocalcin throughout the study. Serum CTX-1 has been previously identified as a reliable indicator of the effects of exercise on the developing skeletal system (McIlwraith, 2005). Concentrations of CTX-1 followed the same trend as osteocalcin, remaining consistent in DRY and H2O while increasing incongruently in CON. Together, these markers indicate that early forced exercise is beneficial for producing consistent bone turnover in young horses, while lack of forced exercise in young horses may lead to inconsistent bone turnover. An increased risk of fracture has been documented in humans during times of increased skeletal growth and increased bone turnover (Parfitt, 1994). Additionally, Lepeule et al. (2013) found that young horses with irregular exercise patterns had an increased risk of osteoarthritic status.

Metacarpal RBAE analysis is a non-invasive method to assess relative bone optical density in individual animals. No treatment or treatment × day effects were observed in the

current study, but there was an effect of day. Both the medial and lateral RBAE increased in all horses throughout the study, which was to be expected as horses were still growing. These results agree with Spooner et al. (2008) which reported that there was no significant change in RBAE of horses who underwent endurance training compared to pasture rearing. This indicates that walking alone is likely sufficient to promote consistent bone turnover, however, may be insufficient to induce phenotypic changes to bone optical density in young horses in only 140 days.

The effect of time on cartilage inflammation and metabolism was driven by a decrease in PGE_2, C2C, C1,2C, and CPII concentrations from d 28 to d 56. This decrease occurred concurrently with increase in bone formation as measured by osteocalcin. The subchondral region of bone and cartilage are related both physiologically and pathophysiologically, working together as one functional unit (Imhof et al., 1999). The current study indicates that there may be a relationship between an increase in bone metabolism and a decrease in response of articular cartilage.

Synovial fluid PGE_2 is well established as an early indicator of joint inflammation and predictor of joint disease (Bertone et al., 2001). However, a lack of change in synovial fluid PGE_2 concentrations in young horses undergoing strenuous exercise has been previously documented (Frisbie et al., 2008), and agrees with the present study, as there were no significant treatment or treatment × day effects on PGE_2 concentrations in synovial fluid. This indicates that the transition from a submaximal exercise program in Phase I to a high intensity exercise program in Phase II does not cause excess inflammation or damage to articular cartilage, even for horses exposed to a reduced load due to buoyancy in an aquatic environment. Furthermore,

this study utilized healthy yearling horses, so it was expected that they would not display any indicators of joint disease.

In regard to articular cartilage, C2C is an indicator of degradation of type II collagen. Following an insult, type II collagen unwinds, exposing epitopes including C2C, which are then released into synovial fluid where they may be detected as a biomarker of collagen metabolism. While there was an effect of time from d 56 to d 140 where C2C concentrations increased in all groups, the highest values reported (136.95 $\pm$ 4.11 ng/mL) are still within range of normal yearling joints reported by Leatherwood et al. (2016; 211.80 $\pm$ 10.17 ng/mL). A more comprehensive indicator of overall cartilage degradation, C1,2C takes into account both type I and type II cartilage degradation. In the current study, no effects of treatment or treatment × day were observed. Frisbie et al. (2008) describe an increase in C1,2C with exercise; however, their animals underwent a strenuous exercise program including trotting and galloping, unlike the submaximal exercise program of the present study, which would not have placed the same degree of force on joints.

Contrastingly, CPII is utilized as a marker of type II collagen formation. It is counter to C2C and is released when an attempt is made to repair type II collagen. There was no effect of treatment or treatment × day, similar to C2C, which was expected as there was no significant degradation to stimulate subsequent regeneration. In response to changes in C2C over time, CPII followed the same trend, decreasing from d 28 to d 56, then increasing from d 56 to completion of the study. This demonstrates the relationship between cartilage formation and cartilage degradation indicative of cartilage turnover. It is important to note that horses of this age are still undergoing significant cartilage turnover, which decreases with age, solidifying the importance of early training for cartilage adaptation to future performance levels.

Additional extraneous factors involved in the introduction of Phase II exercise include change in exercise surface, trajectory of motion, and progressing workload. During Phase I, horses were exercised on treadmills equipped with a synthetic running belt surface. This differed from the natural sand surface of the free-stall exerciser in Phase II; however, the treatment of both exercising groups was consistent throughout. During Phase I exercise, horses tracked in a straight line, then were introduced to a circular track of the free-stall exerciser in Phase II. Similar to the exercise surface, this variable was kept consistent across treatment groups, and exercise was divided evenly in either direction in order to equally distribute joint forces. The introduction of Phase II exercise was gradual, with speeds increasing weekly.

All horses had access to turnout for approximately 10 h/d, where voluntary exercise was allowed. Evidence suggests that voluntary sprinting improves bone optical density and cartilage tissue in weanlings when compared to those housed in stalls (Bell et al., 2001; Billinghurst et al., 2003). While an effort was made to limit size of turnout to prevent the occasional sprint, a sprint of only 50 m, 5 d/wk may be sufficient to increase bone metabolism in young calves used as a model for horses (Hiney et al., 2004). The results observed across all treatment groups during Phase I suggest that turnout and voluntary exercise may be sufficient to support joint health in young horses, similar to walking on a treadmill. Quantification of voluntary exercise would be ideal; however, there are challenges to doing so in horses as the number of steps, gait, speed, and distance are important factors that influence force on bones and joints. Accelerometers are validated using video to determine thresholds for different gaits (Morrison et al., 2015), which may be challenging when measuring multiple individuals in a pasture setting. Additionally, it is difficult to account for high activity levels in young horses, and they are gregarious in nature which may result in loss of device (DuBois et al., 2015). Furthermore, agreement has not been

reached on device placement to obtain the most accurate data (Morrison et al., 2015; Dubois et al., 2015).

The objective of this study was to mimic industry standards of conditioning programs, which typically require 3-4 mo of slow to moderate work followed by 1-2 mo of speed and discipline-specific skill training. Phase I exercise was developed to be comparable to aquatic treadmill protocols utilized in the industry, where exercising horses at speeds higher than a walk is uncommon. If the high intensity exercise program had been extended beyond 28 d, changes in articular cartilage metabolism may have been observed; however, 28 d of intense exercise was sufficient to influence bone generation via serum osteocalcin. No effect was observed in metacarpal RBAE, suggesting the short period of decreased bone formation as indicated by osteocalcin may not have been sufficient to cause outward phenotypic changes to bone density.

CHAPTER VI

CONCLUSION

In conclusion, the results from this study indicate that walking, whether in a dry or aquatic environment, is likely insufficient force to alter molecular regulation of joint metabolism. Early forced exercise, whether in a dry or aquatic environment, was beneficial in producing consistent bone metabolism, while non-exercised horses exhibited incongruent bone turnover in order to maintain a similar phenotypic bone mineral density. However, there were no negative effects of buoyancy on joint development in yearling horses transitioned to an advanced workload, when water was set at 60% of wither height. As water height increases, buoyancy increases and forces on bones and joints decrease (King et al., 2013). Various water heights may be utilized to achieve different objectives for individual animals, but that was not investigated in the current study. Effects of various water heights on bone and joint development may be explored further in the future.

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APPENDIX A

FIGURES

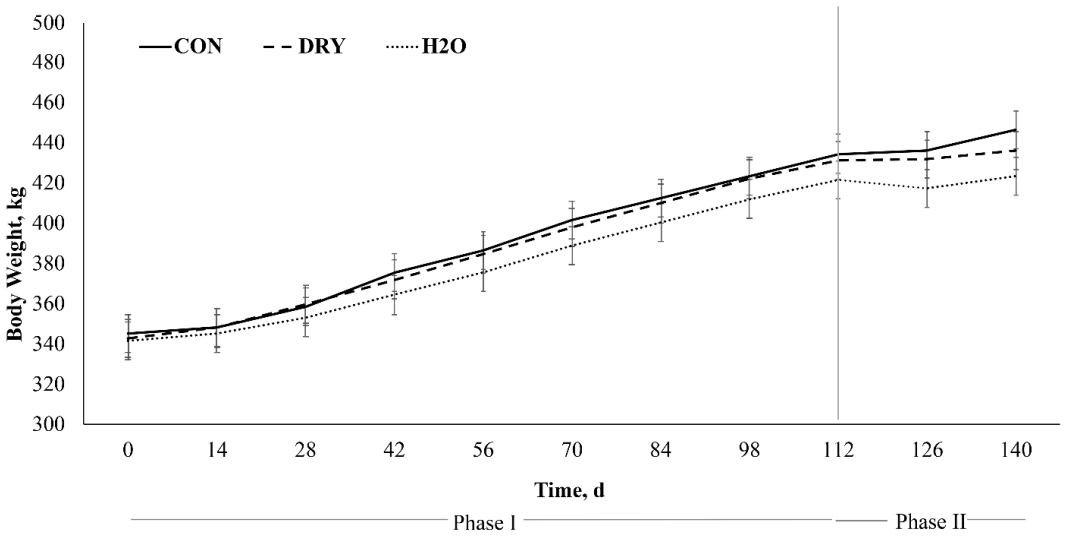

Figure 1. Mean body weight (BW) in kg (least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.67$), day ($P < 0.01$), and treatment × day ($P = 0.12$).

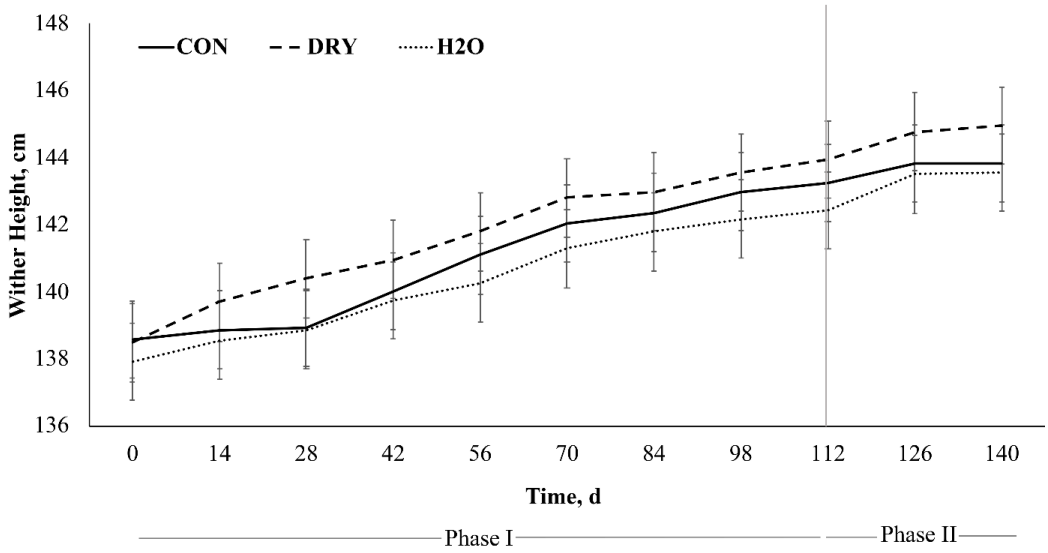

Figure 2. Mean wither height (cm; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.72$), day ($P < 0.01$), and treatment × day ($P = 0.07$).

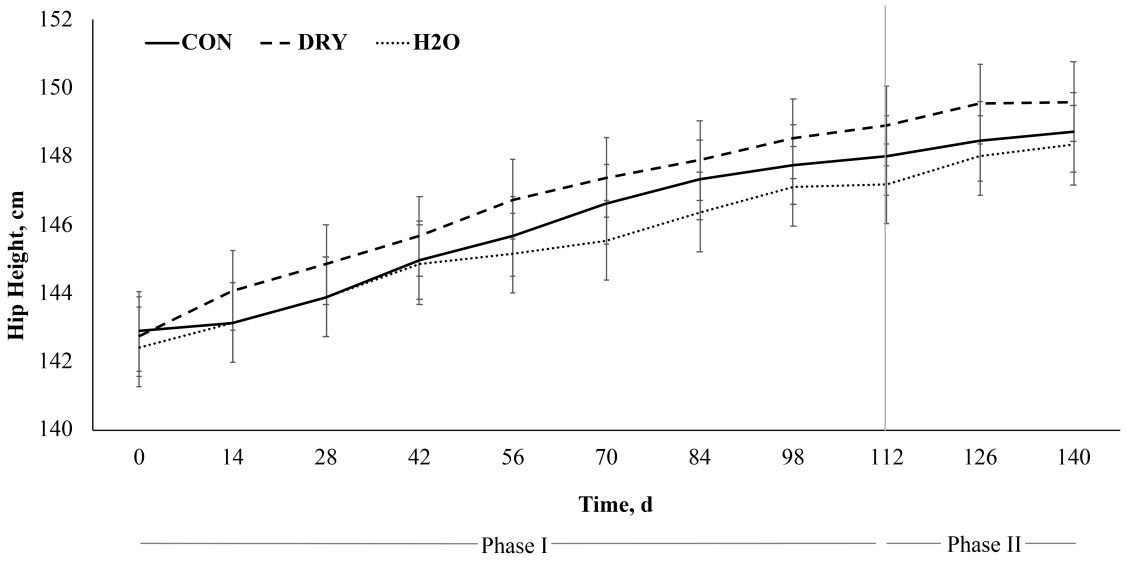

Figure 3. Mean hip height (cm; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.73$), day ($P < 0.01$), and treatment × day ($P = 0.12$).

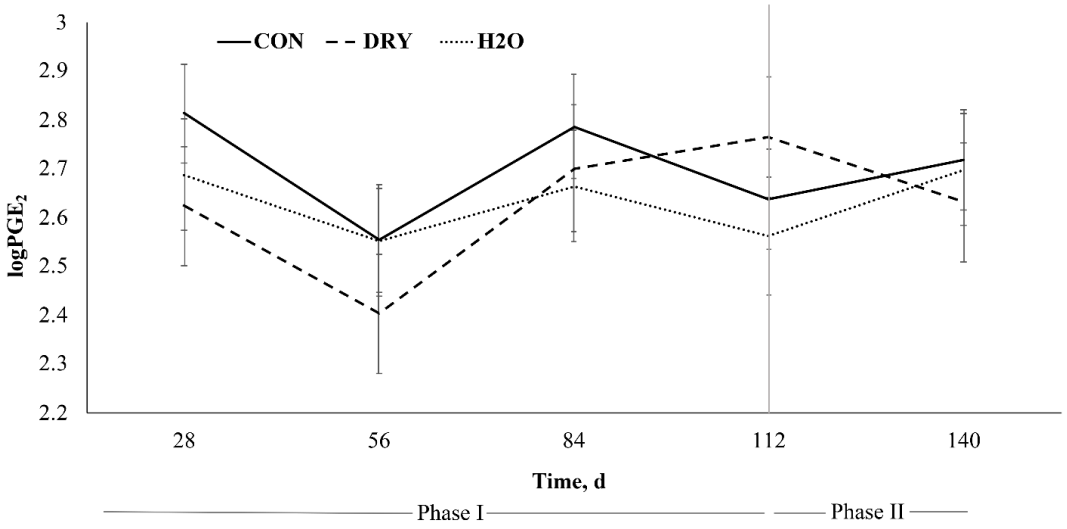

Figure 4. Log of synovial fluid prostaglandin E_2 (PGE_2) concentrations (least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.69$), day ($P < 0.03$), and treatment × day ($P = 0.78$).

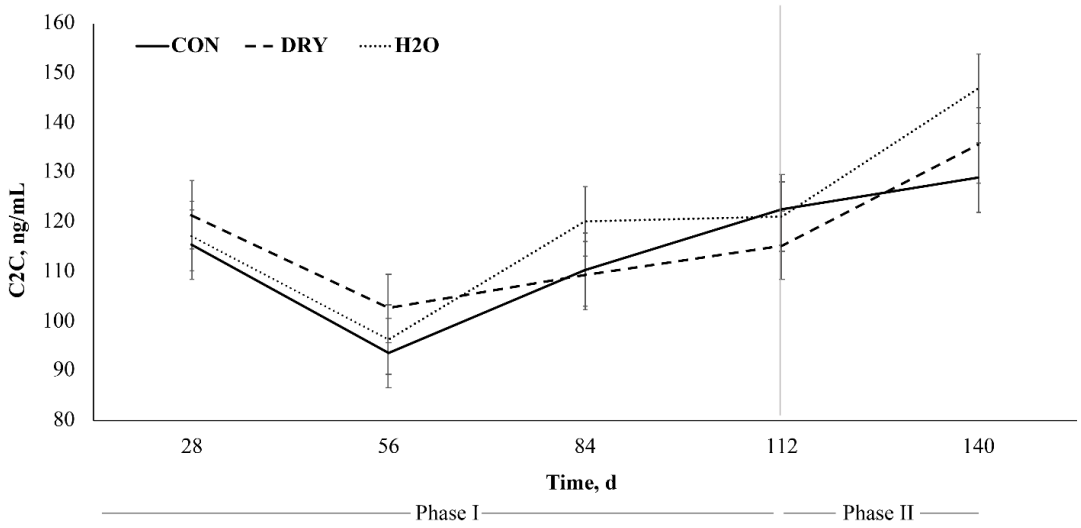

Figure 5. Synovial collagenase cleavage neopeptide (C2C) concentrations (ng/mL; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.65$), day ($P < 0.01$), and treatment × day ($P = 0.59$).

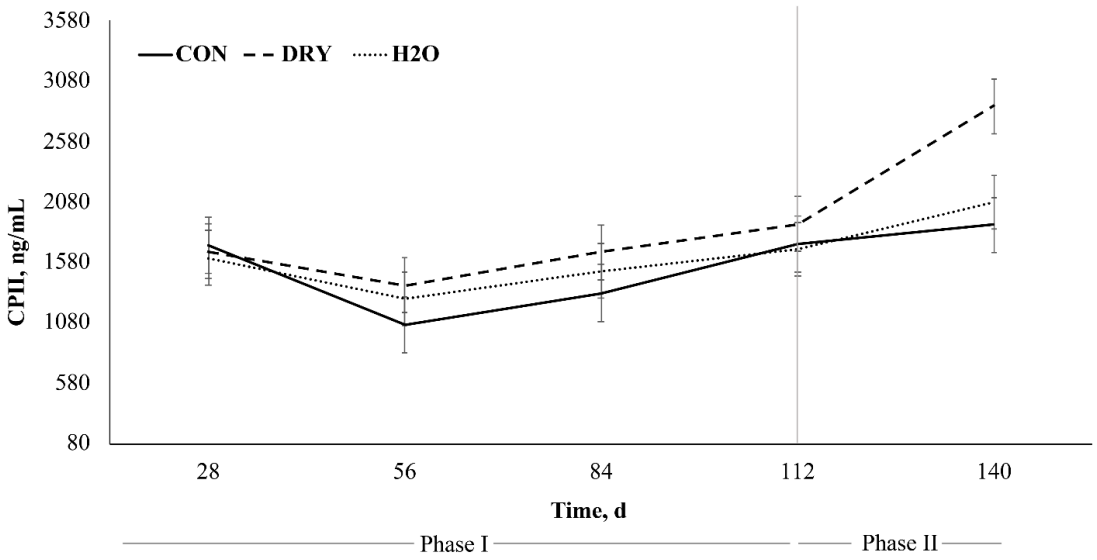

Figure 6. Synovial carboxypeptide of type II collagen (CPII) concentrations (ng/mL; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.30$), day ($P < 0.01$), and treatment × day ($P = 0.21$).

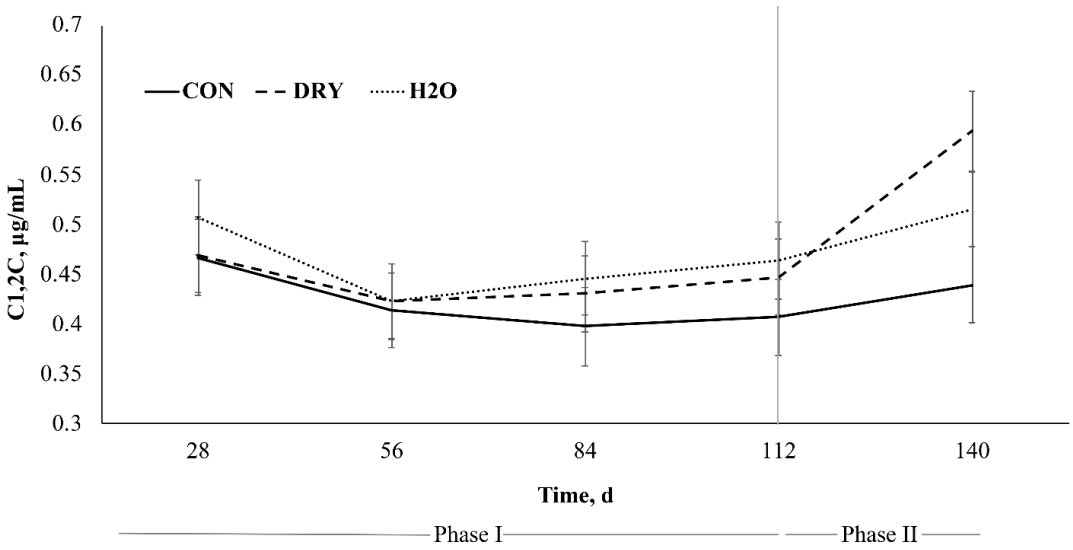

Figure 7. Synovial type I and type II collagen (C1,2C) concentrations (µg/mL; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.35$), day ($P < 0.01$), and treatment × day ($P = 0.45$).

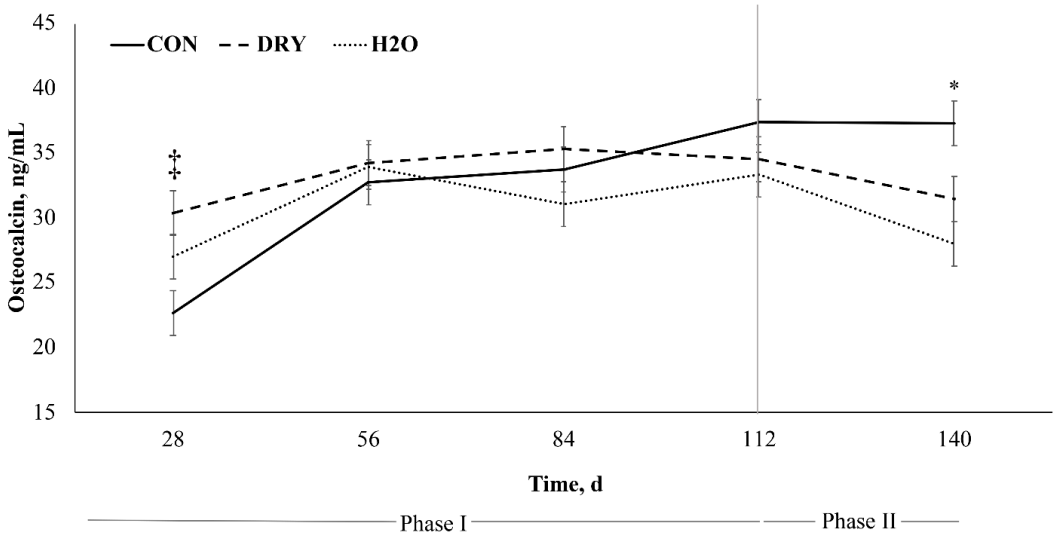

Figure 8. Serum osteocalcin concentrations (ng/mL; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.33$), day ($P < 0.01$), and treatment × day ($P < 0.01$). *Within day, CON differs from DRY and H2O ($P < 0.05$). ‡Within day, CON tends to differ from DRY and H2O ($P < 0.10$).

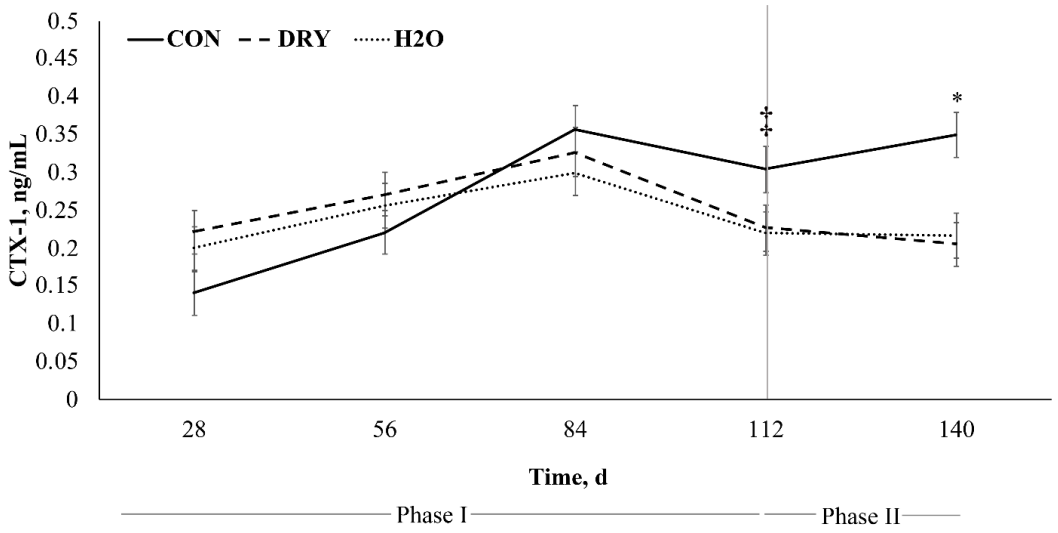

Figure 9. Serum C-telopeptide crosslaps of type I collagen (CTX-1) concentrations (ng/mL; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.37$), day ($P < 0.01$), and treatment × day ($P < 0.01$). *Within day, CON differs from DRY and H2O ($P < 0.05$). ‡Within day, CON tends to differ from DRY and H2O ($P < 0.10$).

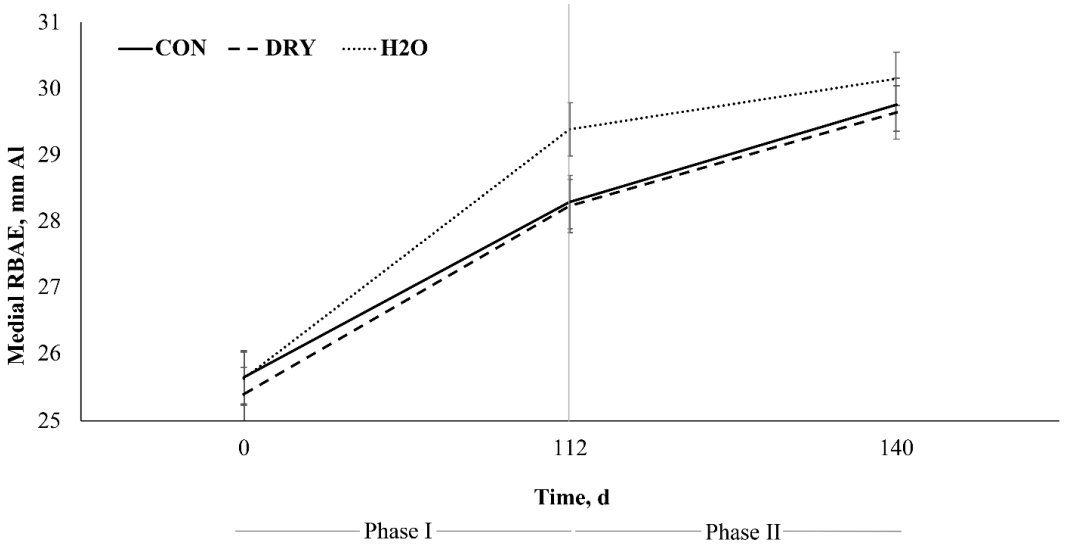

Figure 10. Radiographic bone aluminum equivalence (RBAE) of the medial aspect of the left third metacarpal (mm Al; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.77$), day ($P < 0.01$), and treatment × day ($P = 0.86$).

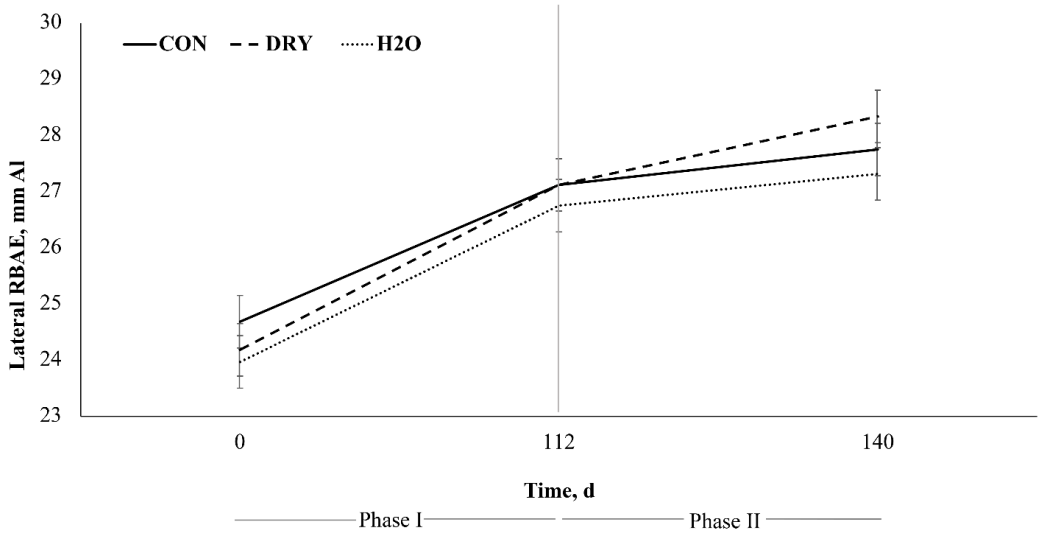

Figure 11. Radiographic bone aluminum equivalence (RBAE) of the lateral aspect of the left third metacarpal (mm Al; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; n = 10), treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (DRY; n = 10), or aquatic treadmill exercise (H2O; n = 10). Main effects include treatment ($P = 0.30$), day ($P < 0.01$), and treatment × day ($P = 0.21$).

APPENDIX B

TABLES

Table 1. Average body weight, average age, number of mares, and number of geldings for each treatment group at allocation on d 0.

	Body Weight, kg	Age, d	Number of Mares	Number of Geldings
CON	341.8	493.7	5	5
DRY	343.0	493.9	4	6
H2O	345.2	500	6	4

Table 2. Exercise protocol for DRY and H2O horses during Phase II (d 112-140).

	Counterclockwise[2]					Clockwise				
Week[1]	2 min	5 min	2 min	5 min	1 min	1 min	5 min	2 min	5 min	2 min
1	1.75	4.0	1.75	4.0	1.75	1.75	4.0	1.75	4.0	1.75
2	1.75	4.0	1.75	6.0	1.75	1.75	4.0	1.75	6.0	1.75
3	1.75	6.0	1.75	6.0	1.75	1.75	6.0	1.75	6.0	1.75
4	1.75	6.0	1.75	6.5	1.75	1.75	6.0	1.75	6.5	1.75

[1]Week of Phase II exercise (d 112-140)
[2]Velocity of free-stall exerciser (Priefert Manufacturing, Mt. Pleasant, TX) in m/s

Table 3. Metabolic biomarkers of cartilage and bone metabolism.

Marker	Abbreviation	Utilization
Osteocalcin	-	Serum biochemical marker of bone formation
C-telopeptide Crosslaps of Type I Collagen	CTX-1	Serum biochemical marker of bone degradation
Prostaglandin E_2	PGE_2	Synovial fluid marker of joint inflammation
Collagenase Cleavage Neopeptide	C2C	Synovial fluid marker of type II collagen degradation
Collagenase of Type I and Type II Collagen	C1,2C	Synovial fluid marker of articular cartilage degradation
Carboxypeptide of Type II Collagen	CPII	Synovial fluid marker of articular cartilage repair